STUDIES ON *IN VIVO* AND *IN VITRO* MYELIN SULFATED GALACTOCEREBROSIDE BIOSYNTHESIS IN CENTRAL NERVOUS SYSTEM TISSUE

By

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In memory of my grandfather,

Curtis M. Sprinkle,

an able teacher and educator for over forty years, and a source of inspiration to me and many other students.

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LIST OF ABBREVIATIONS

KB	α-ketobutyrate, sodium salt
KIC	α-ketoisocaproate, sodium salt
KIV	α-ketoisovalerate, sodium salt
MEM	minimal essential medium (MEM, Eagle-Earle)
PA	phenylacetate, sodium salt
Phe	phenylalanine
PLA	phenyllactate, sodium salt
PSLP	pure solvents lower phase
PSUP	pure solvents upper phase
SBSS-X7	Simm's balanced salt solution (X7)

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The *in vivo* incorporation of radioactive sulfate into CD-1 mouse whole brain, cerebrum, cerebellum, and spinal cord sulfated galactocerebroside as a function of age was determined. Maximum incorporation occurred at approximately 13 days post-partum, after a rapid onset beginning at day 5-8. The ratio of peak incorporation in spinal cord to cerebellum to cerebrum was approximately 12:3:1 respectively, based upon wet weights.

Synthesis of sulfated galactocerebroside was studied *in vitro* in mouse spinal cord culture as a possible model system for the investigation of phenylketonuria and its related metabolite effects upon the synthesis of a specific component of myelin that is formed during early myelination events. Inhibition of net synthesis was observed to the extent of 70 percent at 500 μM phenylpyruvate (PPA) and 50 percent at 1000 μM phenylalanine (Phe) compared to controls. No significant effect was observed at either 300 μM phenyllactate (PLA) or 250 μM phenylacetate (PA). PPA was found to be only slightly inhibitory at 50 μM. The inhibitory effect of PPA was shown

to be reversible when control medium was added back at 21 days *in vitro*.

The extent of L-methionine sulfur contribution to sulfated galactocerebroside synthesis in whole brain *in vivo* was extremely low, only 2600 dpm per gram wet weight after injection of 0.57 μCi L-methionine (^{35}S) at 40 Ci/mM.

Intracerebral bilateral injections were made into CD-1 mice 8-180 days old with Phe, PPA, α-ketobutyrate (α-KB), α-ketoisocaproate (α-KIC), α-ketoisovalerate (α-KIV), PLA and PA, followed by i.p. radioactive sulfate. Sulfated galactocerebroside synthesis was significantly reduced (all $p < .05$) with the first five compounds in mice 8-15 days old, but not with PLA and PA. In adult mice 40-180 days old PPA resulted in a significant decrease in synthesis ($p < .01$), although Phe, α-KB, and α-KIC did show some reduction.

Adult mice also reflected a decrease in their kidney sulfated galactocerebroside synthesis with PPA ($p < .02$), a lesser reduction with Phe, α-KB, and α-KIC, and no change with α-KIV.

The effect of PPA as a structural analog of pyruvate was examined in CD-1 mouse kidney and brain with 1-^{14}C pyruvate, measuring $^{14}CO_2$ release. Both systems, using homogenates, showed decreased $^{14}CO_2$ release. It is suggested that decreases observed in sulfated galactocerebroside synthesis may be due to decreased ATP production required for the synthesis of PAPS, decreased bound galactocerebroside as a result of decreased availability of acetate for synthesis, and general deficits related to oxidative metabolism.

INTRODUCTION

General

Myelin membrane serves as a barrier to extracellular migration and transport of many compounds, including small ions, for example K^+ [1], as well as large compounds. It demonstrates a high electrical resistance, and a low capacitance [2], and the significance of myelin in the increased velocity of conduction in myelinated fibers was experimentally determined in 1966 by Kuffler [3].

Mouse CNS myelin in particular has been shown to contain approximately 25 percent protein, 25 percent phospholipids, 30 to 35 percent cholesterol, and 16 percent sphingolipids. Several functions or possible roles have been attributed to sulfated galactocerebrosides within the latter sphingolipid class of myelin lipids. Sulfated galactocerebrosides as a class are considered to be one of the most stable myelin lipids [4,5]. They have been implicated in cation transport across the myelin membrane [6], assigned a protective role in protease response to myelin basic protein [7], and shown to have a stablizing effect upon myelin membrane [8]. In addition, sulfated galactocerebrosides have shown different patterns in various malignant and virus transformed cells [9], and have been recently shown by Kreps *et al.* [10] to be associated with the specialized function of electrogenesis.

Several methods are available for the biochemical study of myelin metabolism, including direct isolation, purification, and characterization of CNS myelin; *in vivo* methods, usually with postpartum myelin forming animals [11,12], and *in vitro* systems either to assay various enzymes, or by culturing techniques that have recently been made available to study CNS tissue [13-21]. Three of these methods have been applied to study specific effects of phenylketonuria and maple syrup urine disease metabolites upon sulfated galactocerebroside synthesis in mouse CNS tissue.

Metabolism of Sulfated Galactocerebrosides

The synthesis of sulfated galactocerebroside has been shown to proceed according to the following series of reactions:

$$SO_4^{=} + ATP \rightleftharpoons APS + PP_i \qquad (1)$$

$$APS + ATP \longrightarrow PAPS + ADP \qquad (2)$$

$$PAPS + \text{galactocerebroside} \qquad (3)$$

$$\downarrow$$

$$\text{sulfated galactocerebroside} + PAP.$$

The first two reactions have been shown to occur in the soluble phase from 105,000 g centrifugation of whole brain homogenates [22,23]. The active sulfate donor PAPS then transfers the sulfate to the acceptor molecule, galactosylceramide (galactocerebroside) to form sulfated galactocerebroside (sulfatide).

Reaction (3) has been shown to be catalyzed by a cerebroside sulfotransferase enzyme found in the microsomal fraction [24-28]. The synthesis of *in vitro* sulfated galactocerebroside yields the 3-position sulfated product, identical to the natural one [29].

In 1971, Hammarström [30] proposed, based upon GC-MS data, that synthesis of galactocerebroside proceeded via acylation of psychosine (O-galactosylsphingosine). A subsequent paper [31], however, showed that such acylation could occur non-enzymatically and, in fact,proceeded faster in the absence of enzyme. Current evidence [32] supports the ceramide pathway primarily, and little or no firm evidence supports the psychosine pathway to date. The reaction sequence for the synthesis of sulfated galactocerebroside is therefore ceramide → galactocerebroside → sulfated galactocerebroside. The relevant enzymes in the sequence have been purified and characterized [33-37]. The enzyme responsible for the transfer of UDP-gal to HFA-ceramide is present in glial cells, and has been shown by Arora and Radin [38] to be virtually absent from neurons. Reduced activity of this enzyme can lead to reduced sulfated galactocerebroside synthesis and impaired myelin formation as evidenced in msd/u and Jimpy mouse neurological mutants [39,40].

Considerable interest has been generated recently in sulfated galactocerebroside synthesis and degradation in sulfatide lipidosis [41] (metachromatic leucodystrophy). An accumulation of sulfated galactocerebroside occurs not only

in the white matter and gray matter, but also in the kidney. This suggests, but does not prove, that the kidney and brain enzyme are similar or identical. Not much is known about the relationship of sulfated galactocerebroside synthesis in kidney to that in brain. It is clear that the temporal appearance of the cerebroside sulfotransferase enzyme and its activity in kidney are vastly different than in brain. Few comparative studies, however, have looked at chemical compounds that influence brain sulfated galactocerebroside synthesis and relate the findings to the kidney.

Biochemical studies on myelin-specific sulfated galactocerebroside synthesis and other myelination events were delayed for some time due to lack of adequate isolation procedures for the subcellular fractionation of CNS myelin from other structures.

Myelin Isolation and Characterization

Myelin has been isolated from central nervous system tissue by subcellular fractionation techniques, largely by ultracentrifugation [42-58]. Subcellular component marker enzymes such as 2'-, 3'-cyclic nucleotide, 3'-phosphohydrolase and various chemical compounds such as cerbrosides and sphingomyelin have been used to monitor the isolation procedures and to estimate the purity of the fractions obtained [59-66]. Cholesterol, sphingomyelin, plasmalogens, cerebrosides, and sulfated galactocerebrosides (sulfatides) have

been shown to be heavily enriched in white matter, particularly in the myelin sheath. Experiments carried out by Norton and Autilio [45] and Cuzner et al. [44] indicate that approximately 40 to 50 percent of cerebral white matter (dry weight) is myelin. Such myelin isolated by ultracentrifugation commonly contains over 70 percent by dry weight lipid. Virtually all of the remaining dry weight can be accounted for as protein and proteolipid protein.

Prior to the early 1960s, it was widely held that myelin was metabolically inactive and that it had a relatively constant composition at any age in the life of a given animal. Several papers have appeared since that time showing developmental, regional, compositional, and metabolic differences in myelin isolated from various species at different ages [67-79].

Recent advances in subcellular fractionation of myelin membranes, the establishment of reliable markers for the fractions obtained, the development of improved *in vitro* culturing techniques, and the availability of suitable radioactive compounds have made possible the solutions to problems in the biochemistry of the nervous system that were elusive in the recent past. In the present work attempts were made to observe and relate the effects of various metabolites in both *in vitro* and *in vivo* systems upon synthesis of CNS myelin sulfated galactocerebroside.

Present Research

Several major objectives were defined in the present

research. The first was to determine the developmental course, onset, and extent of incorporation of radioactive sulfate into sulfated galactocerebrosides in the developing cerebrum, cerebellum, spinal cord, and whole brain of CD-1 mice. The data obtained served as an *in vivo* control for comparison in subsequent experiments. Secondly, the effects of phenylalanine and the structurally related compounds, phenylpyruvate, phenyllactate and phenylacetate, upon brain sulfated galactocerebroside synthesis *in vitro* were examined using CD-1 mice spinal cord cultures.

Brain analyses of some phenylketonurics, that died of non-neurological causes as adults indicate for the most part a myelin complement of normal composition, suggesting recovery or reversibility from any inhibitory effects upon sulfated galactocerebroside biosynthesis [80]. It seemed, therefore, very important to determine the reversibility or non-reversibility of any severe inhibitory effect observed when cultures were grown in the presence of these metabolites in an *in vitro* system.

The role and extent of methionine sulfur in the early synthesis of sulfated galactocerebroside in mice was investigated using ^{35}S-labeled methionine.

Another objective in the present study was to relate the *in vitro* results back to the intact animal. Intracerebral injections were made into young mice prior to the time of rapid myelination to adulthood using selected phenylalanine-related compounds and α-ketoacids related to conditions in

which CNS myelin deficits have been noted [81-88]. The mice received i.p. $^{35}SO_4^{=}$, and the whole brains were subsequently extracted for sulfated galactocerebrosides.

Attempts were made to relate the brain sulfated galactocerebroside synthesis effects to kidney synthesis based upon the known differences in the temporal appearance of the relevant transferase enzyme.

Finally, a possible explanation is proposed and tested to explain a reasonable basis for the observed inhibitory effects of phenylpyruvate and structurally related compounds upon sulfated galactocerebroside synthesis in mouse kidney and brain.

METHODS AND MATERIALS

Experimental Animals

The experimental animals in the following experiments were CD-1 mice obtained from Charles River. Litter sizes were limited to 8 to 12 pups. Litters outside this range were not used unless otherwise stated in order to control or reduce many of the variables introduced into experiments due to nutritional state, stage of maturation, and general development discussed by some authors [89,90]. The animals were fed commercial lab chow and given water *ad libitum*. Animals were sacrificed by cervical dislocation, decapitation, or over-etherization, depending upon the experiment.

Reagents

Radioactive $Na_2{}^{35}SO_4$, ^{35}S-L-Methionine, [Methyl-^{3}H] thymidine and 1-^{14}C-L-leucine were obtained from New England Nuclear. Phenylpyruvate, L-beta phenyllactic acid, and phenylacetic acid were obtained from Nutritional Biochemicals Corporation. Alpha-ketobutyrate, α-ketoisocaproate, and α-ketoisovalerate were obtained as their sodium salt from Sigma. All acids in the following experiments were used as their sodium salt. Reagent or analytical grade chemicals were obtained from various commercial suppliers.

Sulfated galactocerebrosides were obtained commercially (Applied Science Laboratories, Inc.) or isolated from CD-1 mouse brain by Florisil chromatography [91], DEAE chromatography [92] and thin layer as described subsequently in Methods and Materials.

Radioactive Sulfate Isolation and Counting

Radioactive ^{35}S located in sulfated galactocerebroside, produced in subsequent experiments, was isolated by thin layer chromatography[93,94]and Florisil chromatography [92] and released by hydrolysis in 1 N hydrochloric acid overnight at 105°C in a 15 ml capped centrifuge tube. An equal volume of 1M $BaCl_2$ was added, followed by 4 ml of 1 mg/ml K_2SO_4 solution. The mixture was centrifuged for 15 minutes at 500 g. The precipitate was transferred to a scintillation vial with 2.0 ml of distilled water added in portions. The centrifuge tube was then rinsed with 13 ml Aquasol (New England Nuclear), which was then added to the scintillation vial for counting as a light gel. Recoveries were approximately 95 percent for the precipitation step, and 85 percent was obtained for the counting efficiency. A Packard Tri Carb model 3345 scintillation counter was used to count the radioactive samples. This procedure served as additional confirmation of the identity of the sulfated galactocerebroside produced in the *in vitro* and methionine experiments.

Determination of Phenylalanine-Related Metabolites in Tissue Culture Growth Media by Gas-Liquid Chromatography

Phenylacetate and phenyllactate were estimated by gas-liquid chromatography after extraction from growth medium in which spinal cord cultures were to be fed. One ml of growth medium was acidified to pH 1 with concentrated HCl, and an equal volume of saturated NaCl was added. The aromatic acids were then extracted into diethyl ether or ethyl acetate. The 15 ml screw-capped tubes were centrifuged at 1000 g for 10 minutes after each extraction. Three combined extractions of 5.0 ml each were taken to dryness at 37°C under a steam of nitrogen. Methyl esters were prepared by the addition of 2.0 ml ethereal diazomethane and 2 drops of methanol. After 20 minutes at room temperature, the solvent was removed under vacuum, and the sample was dissolved in an appropriate volume of ethyl acetate for injection. Chromatography was carried out on an F&M 402 gas chromatograph equipped with dual flame ionization detectors with 6 ft x 3 mm i.d. glass columns packed with 3 percent OV-17 liquid phase on 80/100 mesh Chromosorb G (Applied Science Laboratories, Inc.). Instrument parameters were: air 400 cc/min, hydrogen 50 cc/min, nitrogen 75 cc/min, detector 275°C, injection port 250°C, temperature 160°C isothermal, or linearly programmed 120 to 180°C. No methyl phenylpyruvate was detected, and no appreciable 2, 4-dinitrophenylhydrazone could be isolated from 1 ml medium. Peak areas were measured by triangulation for quantitation.

Fluorometric Analysis of Phenylalanine in Tissue Culture Growth Medium

Phenylalanine levels were measured in serum and growth medium by the method of McCaman and Robins [95] using a Turner model 111 fluorometer. Aliquots of 200 µl of serum or media were combined with 200 µl of 0.6 N TCA, mixed well, and centrifuged after 10 minutes. Samples of 50 µl were withdrawn for analysis.

In Vitro Growth Medium

The growth medium for the spinal cord culture experiments consisted of either Medium A or Medium B, whose compositions are listed in Table 1. Growth medium B is a modification of one developed by Edith R. Peterson and used in the laboratory of Dr. Murray Bornstein. Explants of CD-1 mouse cord were grown in a lying drop position in Maximow chambers contained in vertical level racks in a Hotpack incubator at 35°C.

Isolation of Mouse CD-1 Spinal Cord for *In Vitro* Studies

Fourteen and one-half-day pregnant CD-1 mice were sacrificed by cervical dislocation and the abdomen was soaked in 95 percent ethanol for 10 minutes. Embryos were carefully removed using sterile stainless steel scissors and forceps and transferred to a 100 x 15 mm petri plate containing Eagle-Earle Minimal Essential Medium (MEM) supplemented to

TABLE 1

TISSUE CULTURE GROWTH MEDIUM

A. 6.0 ml SBSS - X7
4.0 ml Fetal Calf Serum
0.12 ml 50% glucose
0.10 ml 200 mM glutamine
50 units / ml penicillin

B. 5.1 ml Minimal Essential Medium (MEM, Eagle - Earle)
1.0 ml Chick Embryo Extract
3.4 ml Human Cord Serum
0.12 ml 50% glucose
50 units / ml penicillin
0.10 ml 200 mM glutamine
1.0 µg / ml ascorbate

approximately 600 mg percent glucose and 100 units/ml penicillin. Further dissection was done under a binocular microscope using only Dumont #5 stainless steel forceps and #11 surgical blades mounted in #5 stainless steel handles. The spinal cord was carefully dissected out using an initial dorsal and dorsolateral approach and stripped of adjacent tissue and coverings. Ganglia were removed. The spinal cord was then bathed in freshly prepared growth medium and cut into sections less than 1 mm thick for explantation. Care was exercised throughout to minimize trauma to the cord, and the distal portions of each cord were discarded. If sections were over 1 mm in thickness, a large necrotic zone was found and very thin sections resulted in failure to myelinate. Two-tenth mm background grids were therefore used to obtain uniform optimum sections. Hemisections of cord were transferred after each cord was sectioned into freshly prepared growth medium using a wide-bore pipette that had been fire polished and sterilized. Sections were then transferred to collagen-coated coverslips that had been preconditioned in medium as described above. Cultures were routinely fed 100 µl medium 2 to 3 times a week, and kept in Maximow chambers at 35°C in the lying drop position. Additions to the growth media of phenylalanine, phenylpyruvate, phenyllactate, and phenylacetate were made and are listed in Table 2.

Isolation of Central Nervous System Myelin by Ultracentrifugation

Myelin was extracted from brain tissue in these experiments

TABLE 2

Tissue culture medium content of phenylalanine, phenylpyruvate, phenyllactate and phenylacetate before and after additions of these compounds

Compound[a]	Medium before additions	Expt 1[b]	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
Phenylalanine[c]	45.8 ± 1.8μM (n=5) (s. e. m.)	100μM	1000μM	-----	-----	-----	-----
Phenylpyruvate	<2μM	-----	-----	50μM	500μM	-----	-----
Phenyllactate	<2μM	-----	-----	-----	-----	300μM	-----
Phenylacetate	<2μM	-----	-----	-----	-----	-----	250μM

a Analyzed by gas-liquid chromatography as described in methods and materials.

b All media were adjusted to these final concentrations for feeding cultures.

c Fluorometric determination.

using a modified procedure of Cuzner and Davison [44,55]. Osmotic shock of the myelin after preliminary isolation was carried out to remove loosely bound proteins. Myelin isolated in this manner was used as cold carrier myelin in *in vitro* experiments and as a source of reference sulfated galactocerebroside.

Collagen Matrix Preparation

Rat tails stored at -20°C from six 250-gm Sprague-Dawley rats were soaked in 95 percent ethanol for 10 minutes. The skin of each tail was dissected free and the silvery white tendons were carefully dissected out and transferred to distilled water for rinsing. After rinsing with several changes of distilled water, the tendons were extracted into 500 ml sterile 1:1000 acetic acid in deionized water for 24 to 48 hours and then centrifuged in 250-ml sterile bottles at 10,000 g for 1 hour in a Sorvall RC 2-B centrifuge at 4°C. The upper layer was carefully decanted off into sterile 100 ml bottles for storage. The viscous collagen solution was stable at 4 to 10°C for several months.

Immediately before use, 10 ml collagen solution was dialyzed against 1800 ml sterile water in autoclaved dialysis tubing (1/4 to 1/2 inch diameter) overnight at 4°C. A water change was made the next morning with 1 ℓ of sterile water. Dialysis was continued until the collagen became viscous such that an inverted dialysis bag produced retarded air

bubble flow upward. Over-dialysis produced a gel that was difficult to use for a growth matrix. Dialyzed collagen solution was added to a 7/8-inch coverslip or other suitable growth surface and solidified to a gel by exposure to ammonia vapor. The collagen-coated surface was then neutralized by several rinses of sterile .01 to .02 percent phenol red solution and preconditioned in Eagle-Earle MEM supplemented to 600 mg percent glucose and 100 units/ml penicillin. The collagen matrix was conditioned for at least 48 hours prior to use in the proper growth medium.

Undialyzed collagen solution, lyophilized, contained approximately 66 to 70 percent protein by weight as determined using the Lowry procedure [96] and contained approximately 300 to 500 µg protein/ml in the solution.

DNA and Protein Synthesis and Isolation from Mouse Spinal Cord Cultures

Cultures of CD-1 mouse spinal cord were labeled with 1 µCi/ml each of thymidine [methyl-^{3}H] at a specific activity of 20 Ci/mM and 1-^{14}C-L-leucine at a specific activity of 53.5 mCi/mM. Cultures were rapidly rinsed in cold Simm's balanced salt solution (SBSS-X7) supplemented to 600 mg percent glucose. Cultures were pooled, homogenized in 1 ml 0.9 percent saline at 4°C using a Teflon-glass homogenizer and 3.5 ml cold TCA was added to a final concentration of 5 percent. DNA and protein were then isolated by centrifugation for 20 minutes at 13,500 g and 4°C. The pellet was

resuspended in cold 5 percent TCA twice, spun down as above, solubilized in NCS solublizer (New England Nuclear) and counted in toluene-based scintillation fluid.

Lipid Extractions

Lipids were extracted by the Folch-Pi, Lee, and Sloan-Stanley procedure [97]. A 0.2 volume of 0.37 percent K_2SO_4 was used to form the two-layer system, and after centrifugation at 500 g for 10 minutes, the upper layer was aspirated. The lower phase was brought up to a volume of 8.0 ml with PSLP. The solution was then rinsed 2 to 4 times with half volumes of PSUP until the last rinse top layer contained fewer counts than twice background. The two-phase system was briefly centrifuged (5 min) after each rinse. The final lower phase was transferred to glass scintillation vials, where the solvent was removed by heat. When the vials were almost dry, they were placed under a gentle stream of nitrogen. Ten ml Aquasol was added and the samples were counted on a Packard Tricarb model 3345 scintillation counter.

In Vivo Synthesis and Distribution of Sulfated Galactocerebroside Radioactivity in Whole Brain, Cerebrum, Cerebellum and Spinal Cord

CD-1 mice at various ages were injected i.p. with 0.57 μCi $Na_2{}^{35}SO_4$ at a specific activity of 850 mCi/mM and specific regions of brain were isolated and extracted for lipid soluble counts as described above.

Methionine ^{35}S Sulfur Incorporation into Whole Brain Sulfated Galactocerebroside-Specific Radioactivity

CD-1 mice of various ages were injected i.p. with 0.57 μCi ^{35}S-L-methionine at a specific activity of 40 Ci/mM. Whole brain was extracted 24 hours later for lipid soluble radioactivity after sacrifice by over-etherization. After lipids were extracted as described above, the extracts were dried under vacuum at 60°C, redissolved in a small volume of 2:1 chloroform-methanol, and subjected to TLC as described below.

Thin Layer Chromatography

Thin layer sulfated galactocerebroside separations were done on Silica Gel G plates with or without binder on plates 100, 250, or 500 microns thick. Solvent systems used were:

(a) 75:25:4 (chloroform-methanol-water),

(b) 65:25:4 (chloroform-methanol-water), and

(c) 5:4 (chloroform-methanol) or (b) and (c).

Plates were developed in equilibrated paper-lined TLC tanks (Brinkmann).

In Vivo Sulfated Galactocerebroside Synthesis in the Presence of Added Compounds

CD-1 mice of various ages were injected intracerebrally and bilaterally under ether anesthesia with 8.0 μg of either PPA, Phe, PLA, PA, α-KB, α-KIC, or α-KIV as their sodium salt.

Control animals received 0.9 percent saline. Within 15 minutes of the intracerebral injections of 1 µl volume, each mouse received 0.57 µCi/mM $Na_2{}^{35}SO_4$ i.p. at an activity of approximately 215 mCi/mM. Sulfated galactocerebroside counts were extracted from whole brain and kidney after periods up to 24 hours as described under Methods and Materials, and combined brain extracts were counted as lipid soluble counts. Two-dimensional TLC was carried out on random brain extracts to be sure that the differences in observed counts did, in fact, reflect differences in sulfated galactocerebroside synthesis, and not some other lipid soluble sulfur-containing compound. In each and every case, the changes in counts reflected a corresponding change in sulfated galactocerebroside-specific counts. All kidney extracts were subjected routinely to two-dimensional TLC in the presence of 25 µg cold carrier sulfated galactocerebroside as described in Methods and Materials, and visualized by exposure to iodine vapor. The iodine was allowed to sublime off and the spots were counted in Aquasol.

Measurement of $^{14}CO_2$ Release from 1-^{14}C Labeled Pyruvate in Mouse Kidney and Brain Homogenates

Brains and kidneys from five adult CD-1 mice (40 to 180 days of age) were homogenized in 0.25 M sucrose. One ml brain and 1 ml kidney homogenate containing 13 and 25 mg protein, respectively, as measured by the biuret reaction, were added to 4.0 ml Krebs phosphate buffer at pH 7.4 in 25-ml

reaction flasks. Each flask was gassed with oxygen, fitted with a Hyamine well that would hold approximately 0.6 ml and incubated capped at 37°C. Five minutes later, radioactive substrate at 300,000 cpm and 1 x 10^{-3}M, and PPA, KIC or KIV at various concentrations were added in a volume of 1 ml, injected into the flasks. After gently shaking the flasks for exactly 30 minutes, 1 ml 1 N H_2SO_4 was injected into each flask. One hour later, the 0.4 ml Hyamine from each CO_2 trap was quantitatively transferred to 10 ml toluene-based Omnifluor scintillation fluid and counted.

RESULTS AND DISCUSSION

In Vivo Synthesis of Sulfated Galactocerebrosides in Whole Brain, Cerebrum, Cerebellum, and Spinal Cord

The incorporation of radioactive sulfate into sulfated galactocerebrosides has been studied by several authors in rats and mice [24-28,70,97-98]. A maximum rate of accumulation of sulfated galactocerebrosides has been shown to occur approximately 17 days after birth in rats [97,99-100] and in mice [101]. Little is known about the regional distribution of the radioactivity, since most previous studies have utilized whole brain extracts.

In this study, CD-1 mice were used in order to determine accurately the onset, period of maximum incorporation, and the regional distribution of that sulfated galactocerebroside radioactivity as a function of age in the cerebrum, cerebellum, and spinal cord. The results of i.p. injection of $Na_2{}^{35}SO_4$ and subsequent isolation of lipid-soluble sulfated galactocerebroside counts in whole brain are shown in Figure 1. It has been shown by several authors [27,97,100] that sulfur radioactivity from $Na_2{}^{35}SO_4$ injected during the period of rapid myelin synthesis is found specifically in sulfated galactocerebrosides to the extent of approximately 90 percent or more. This fact was confirmed in the present

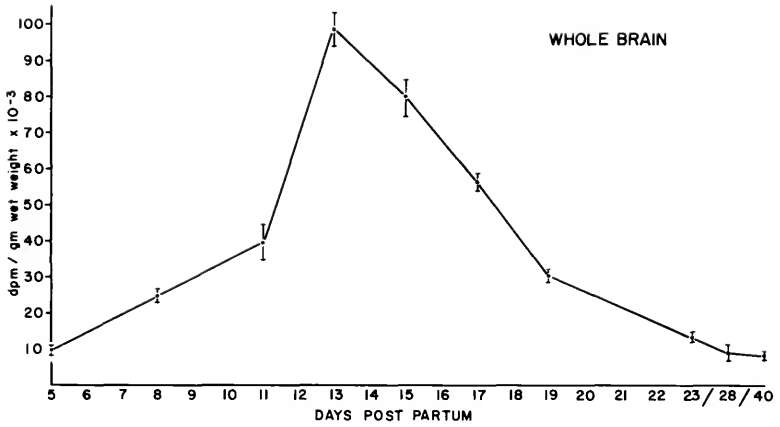


Figure 1. *In vivo* age profile of $^{35}SO_4^=$ injected i.p. and incorporated into CD-1 mouse whole brain lipid-soluble radioactivity. Over 90 percent of the radioactivity was found specifically in sulfated galactocerebrosides, therefore lipid-soluble counts in this and subsequent experiments are taken to reflect synthesis of that compound directly unless stated otherwise. Five mice were each injected with 0.57 μCi/gm body weight of $Na_2{}^{35}SO_4$ at an activity of 850 mCi/mM for each data point as described in Methods and Materials. Values are reported as means ± s.e.m.

experiments by TLC in several solvent systems, and by column chromatography as described in Methods and Materials. Lipid soluble counts were considered, therefore, to reflect sulfated galactocerebroside counts directly unless stated otherwise.

A rapid onset of incorporation of $^{35}SO_4^=$ was found beginning on or about days 8 to 11 postpartum. At least two enzyme systems must be fully functional by this time; the PAPS generating system [26,89,97] involving the cytosol sulfurylase and kinase steps, and the relevant membrane-bound transfer enzyme, cerebroside sulfotransferase [24]. Maximum incorporation of $^{35}SO_4^=$ occurred between 12 and 14 days after birth. Adult animals incorporated $^{35}SO_4^=$ at a level comparable to 5-day-old animals. Recent evidence [70,102] supports the existence of two brain sulfate pools, one large pool associated with sulfated galactocerebrosides with turnover half-times on the order of 9 months or a year, and another very small pool for the synthesis of sulfated galactocerebrosides with a turnover half-time on the order of 2 1/2 days. Evidence suggests that the latter pool is involved in the outermost myelin layer synthesis. Presumably the incorporation observed in the adult animals reflects a composite of continuing new stable myelin sulfated galactocerebroside synthesis and that of the more metabolically active outer membrane regions. Each data point shown in Figure 1 represents the mean of five animals, extracted independently as described in Methods and Materials. The peak of incorporation

in whole brain occurred in this experiment at 13 days post-partum (dpp) and reached a level of 100,000 dpm/gm wet weight brain. Litter sizes in these experiments were limited to 8 to 12 pups per litter, for reasons cited earlier.

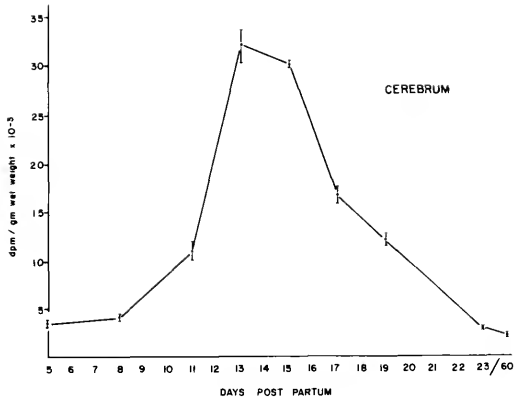
The regional distribution of the sulfated galactocerebroside radioactivity in the cerebrum, cerebellum, and spinal cord as a function of age are shown in Figure 2. All injections were made at 0.57 μCi/gm body weight, and at the same specific activity so that the data are directly comparable.

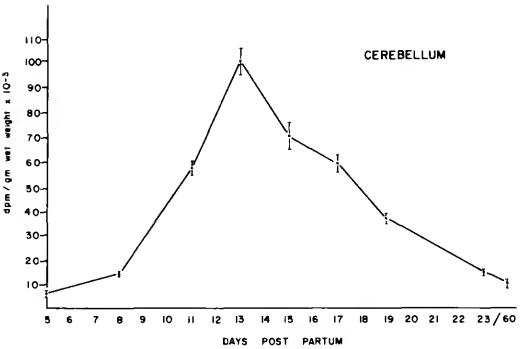
The age at which maximum incorporation of $^{35}SO_4^=$ occurred into sulfated galactocerebroside in CD-1 mice was exactly the same (13 dpp) for cerebrum, cerebellum, and spinal cord under the present experimental conditions. The levels of incorporation in cerebrum, cerebellum, and spinal cord at day 13 were found to be approximately 32,500, 100,000, and 380,000 dpm/gm wet weight tissue, respectively. These results correlate well with histological studies showing increases in stainable myelin during this period. Cerebrosides and sulfated galactocerebrosides are considered by many authors as a result of these studies to be among the most reliable indexes of myelin mass in the central nervous system [55,76, 78,103-107].

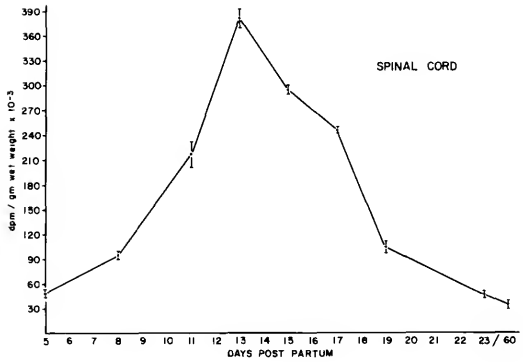
Methionine Contribution to Sulfated Galactocerebroside Synthesis

The specific contribution of methionine sulfur to sulfated galactocerebroside synthesis is shown in Figure 3. The

Figure 2. Distribution of lipid soluble radioactivity as sulfated galactocerebroside from i.p. administered $^{35}SO_4^=$ in CD-1 mouse cerebrum, cerebellum, and spinal cord as a function of age. Details are described in the text. Each point represents average of 5 animals. Results are expressed as means ± s.e.m.


CEREBRUM
dpm./ gm. wet weight x 10-3
35
30
25
20
15
10
5
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23/60
DAYS POST PARTUM


CEREBELLUM
dpm./ gm. wet weight x 10-3
110
100
90
80
70
60
50
40
30
20
10
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23/60
DAYS POST PARTUM


SPINAL CORD
dpm./ gm. wet weight x 10-3
390
360
330
300
270
240
210
180
150
120
90
60
30
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23/60
DAYS POST PARTUM

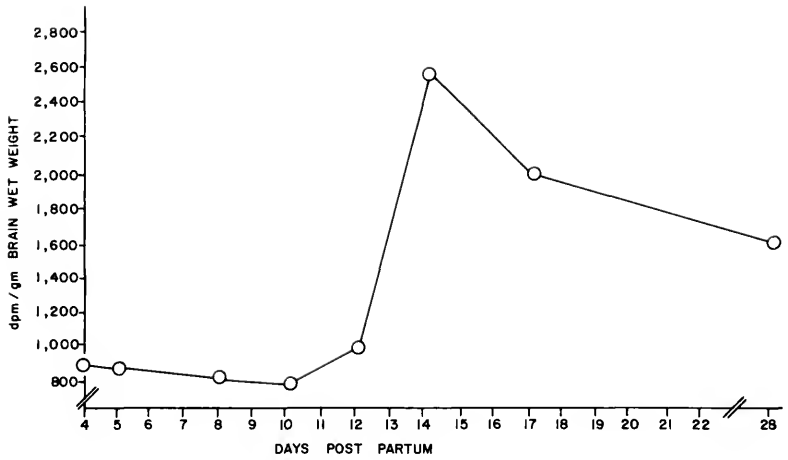


Figure 3. Extent of the contribution of ^{35}S-methionine sulfur in the *in vivo* synthesis of sulfated galactocerebrosides in CD-1 mice. Each point is the mean of 4-5 mice. Each animal recieved 0.57 μCi/gm body weight of ^{35}S-L-methionine at a specific activity of 40 Ci/mM. Lipid extracts from each age animal were combined and subjected to 2-dimensional TLC to separate sulfated galactocerebrosides from methionine-containing lipids.

open circles indicate the dpp age of the mice at the time of injection. The results are expressed as mean dpm ^{35}S as sulfated galactocerebroside per gm wet weight whole brain. Radioactive product was obtained completely separated from methionine-containing lipids. The delay in peak incorporation compared to Figure 1 probably represents the increase in time required to oxidize the sulfur to $^{35}SO_4^{=}$.

Elevated levels of methionine have been noted in the blood of homocystinurics as well as elevated levels of homocystine [108] and questions have been raised as to the effects of that methionine upon sulfate utilization in brain. Subcutaneous injections of methionine in rats followed by i.p. $Na_2{}^{35}SO_4$ have been shown to result in a marked decrease of label incorporation into sulfated galactocerebrosides compared to controls [100]. It is apparent in Figure 3 that only 2600 dpm ^{35}S was incorporated at the peak (day 14) in CD-1 mice using i.p. injections. Direct intracerebral injections produce similar results. It was, therefore, concluded that the methionine sulfur atom was nc utilized extensively in sulfated galactocerebroside synthesis under the present experimental conditions. However, one cannot exclude the possibility that methionine may influence the sulfate available for synthesis, particularly if the levels in blood remained elevated, by effecting entry of sulfate into the cell.

In Vitro Synthesis of Sulfated Galactocerebrosides in Mouse Spinal Cord Cultures

Mouse spinal cord cultures were grown in Maximow chambers as described in Methods and Materials in the presence of $Na_2{}^{35}SO_4$. After various periods of time, cultures were harvested and extracted for sulfated galactocerebroside radioactivity in myelin. The results, shown in Figure 4, indicate increased incorporation of radioactivity into myelin through 32 days *in vitro*. Non-radioactive myelin used as carrier was isolated and purified according to the flow diagram in Figure 5.

The Effects of Phe, PPA, PLA, and PA Upon *In Vitro* Sulfated Galactocerebroside Synthesis

The *in vitro* system was then utilized to determine the effects of phenylalanine, phenylpyruvate, phenyllactate and phenylacetate upon ^{35}S incorporation into myelin-specific sulfated galactocerebroside as a system in which to study conditions where general myelin deficits have been observed. Additions of these compounds were made to control medium according to the levels listed in Table 2. Marked inhibition was found in the case of 1000 µM Phe and 500 µM PPA as shown in Figure 6a, and little or no inhibition was observed at any of the concentrations used of PA or PLA (Figure 6a or 6b). The reduction in $^{35}SO_4{}^{=}$ incorporation into sulfated galactocerebrosides was approximately 50 percent at 1000 µM Phe, and 70 percent at 500 µM PPA. The results suggest, but

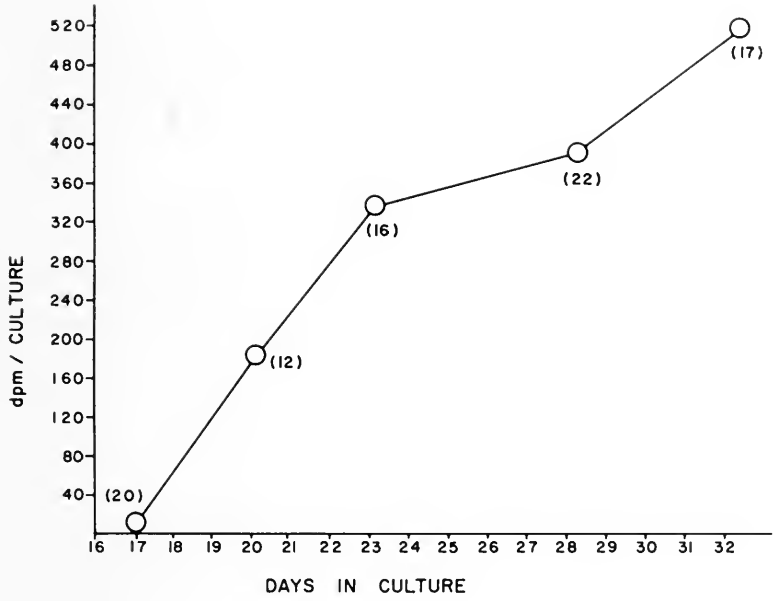


Figure 4. *In vitro* incorporation of $^{35}SO_4^{=}$ into myelin-specific sulfated galactocerebrosides in CD-1 mouse spinal cord cultures. Hemisections of spinal cord were grown in the presence of approximately 100,000 cpm of $Na_2{}^{35}SO_4$ at an activity of 850 mCi/mM for 3 days before harvesting. Cultures were harvested at various time intervals and co-migrated with purified cold carrier myelin and extracted for sulfated galactocerebroside-specific radioactivity.

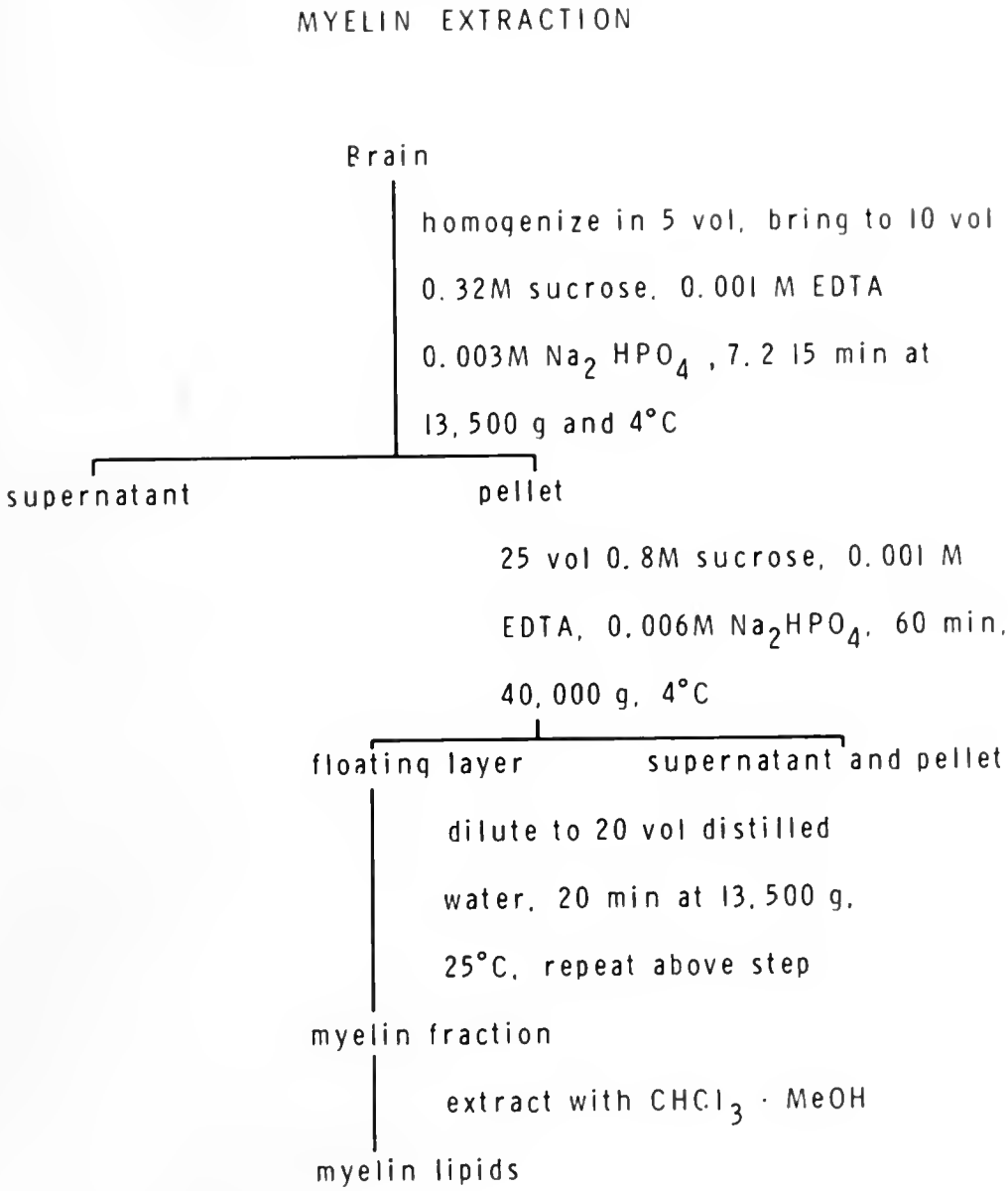


Figure 5. Myelin isolation and extraction. Ninety-five to 99 percent of the preparation by weight was soluble in 2:1 chloroform-methanol, and the myelin obtained was used as cold carrier, in the previous experiment and in subsequent experiments, to isolate sulfated galactocerebroside reference standards.

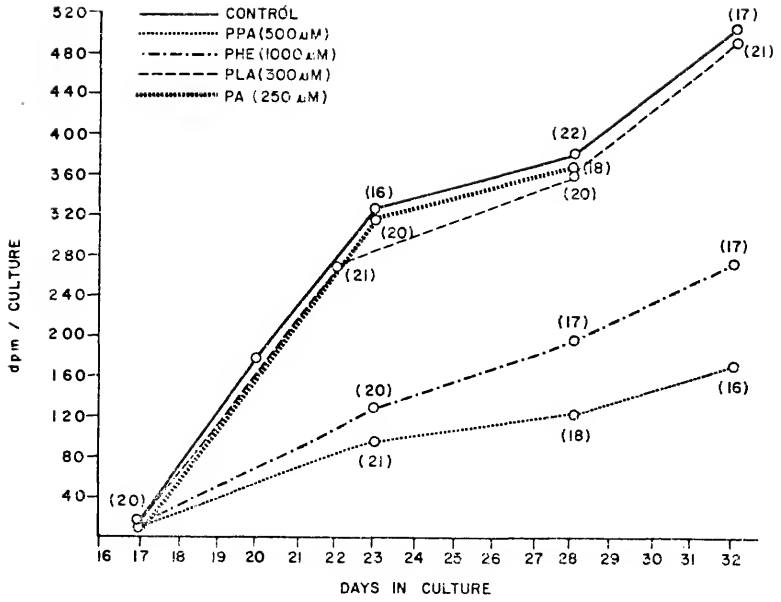


Figure 6. The effect of phenylalanine, phenylpyruvate, phenyllactate, and phenylacetate upon sulfated galactocerebroside synthesis in CD-1 mouse spinal cord culture at high (a) and low (b) concentrations. Compounds were added at the time of explantation at concentrations shown in Table 2. The cultures were grown various lengths of time and myelin-specific product was isolated as described in Methods and Materials. The number of cultures represented by each data point are indicated in parentheses.

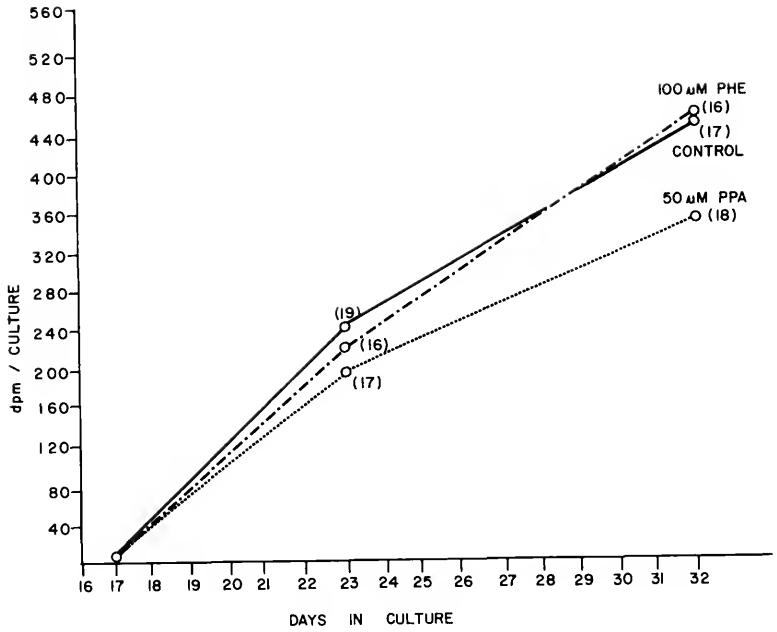


Figure 6 continued

do not prove, that phenyllactate is not appreciably oxidized under the present experimental conditions. It may also be possible to correlate the level of PPA in the Phe curve with the observed effects in order to obtain better insight into the nature of the inhibition.

The Effect of PPA Removal upon Sulfated Galactocerebroside Synthesis *In Vitro*

Spinal cord cultures grown in the presence of 500 µM PPA were rinsed at 21 days in culture with SBSS-X7 at 600 mg percent glucose and fed control medium out to 32 days in culture. At 26 and 32 days in culture, spinal cord explants were removed from the incubator and extracted as described previously. The results in Figure 7 indicate a 74 percent recovery of control values at 32 days in culture. Radioactive product in these experiments was chromatographed in one- and two-dimensional TLC systems in order to offer additional confirmation of the structure. Figure 8 illustrates such an isolation in a 75:25:4 solvent system. Visualization was done by exposure to iodine vapors. Silica Gel was scraped from the plates and counted in Aquasol. The radioactivity corresponded to the position of authentic carrier. The distances from the origin are indicated in mm. The myelin-specific radioactivity obtained co-migrated with authentic sulfated galactocerebroside as shown in Figure 9 in 75:25:4, 65:25:4 (chloroform-methanol-water), and 5:4 chloroform-methanol systems, as well as in benzene-methanol (7:3), and by

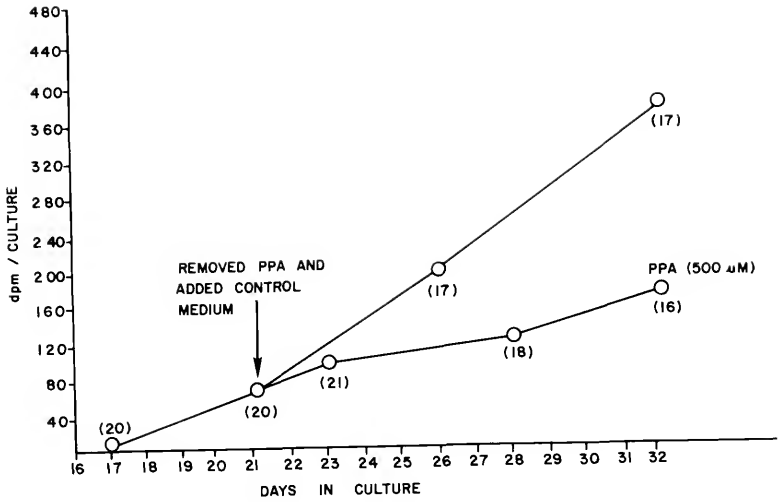


Figure 7. The effect of phenylpyruvate removal and replacement with control growth medium in CD-1 mouse spinal cord culture upon net sulfated galactocerebroside synthesis. PPA was added at the time of explantation.

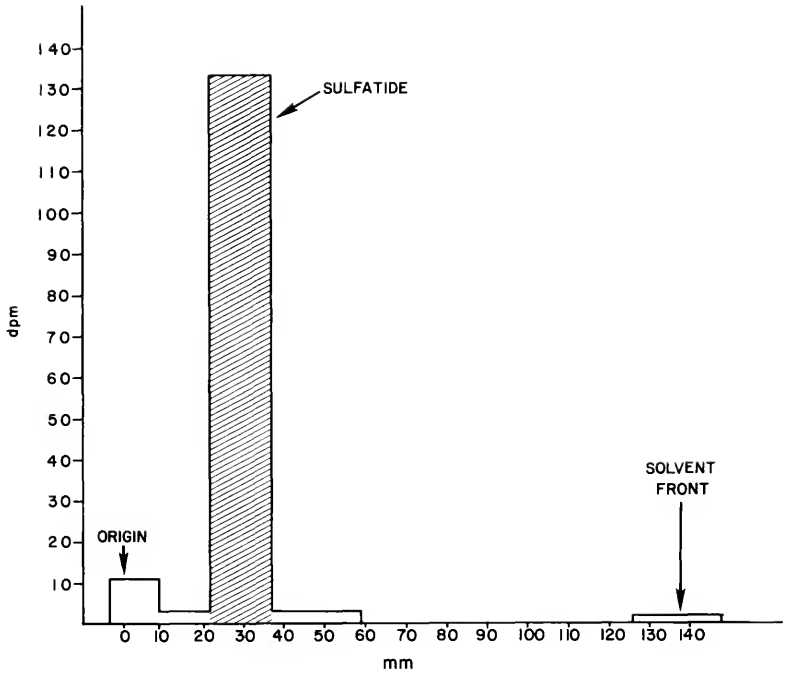
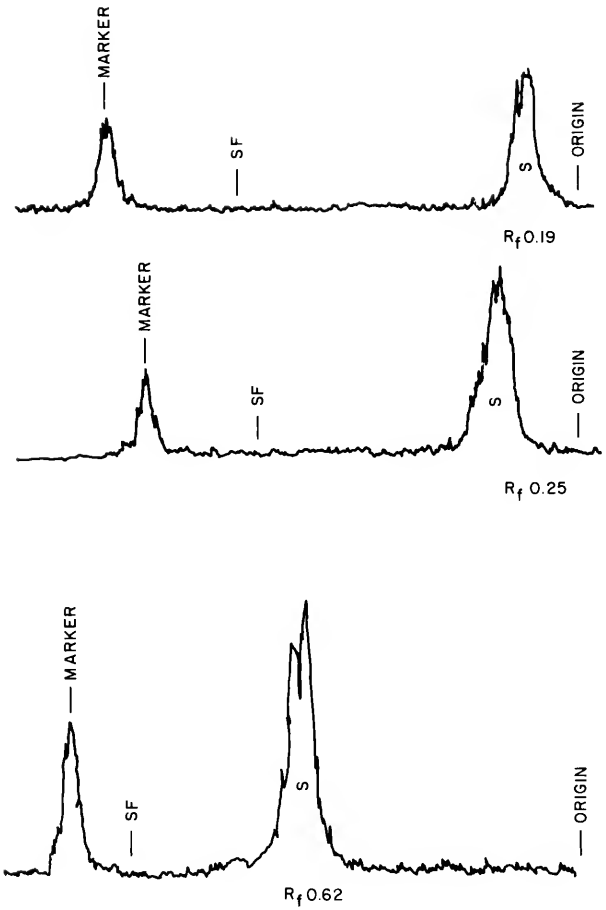


Figure 8. Thin layer chromatography of radioactive myelin-specific sulfated galactocerebrosides isolated from cultured CD-1 mouse spinal cord. Twenty cultures (17 days *in vitro*) were grown as described previously in Figure 4, homogenized in sucrose and co-migrated with approximately 10 mg unlabeled purified myelin by ultracentrifugation. The ^{35}S-labeled product was isolated by 2:1 chloroform-methanol extraction and then chromatographed on Silica Gel G plates using a 75:25:4 chloroform-methanol-water solvent system.

Figure 9. Radiochromatogram of isolated *in vitro* synthesized sulfated galactocerebroside. The R_f values correspond to authentic sulfated galactocerebrosides run under the same conditions (75:25:4, 65:25:4, and 5:4 in order of increasing R_f values in chloroform-methanol-water systems) cold carrier myelin extract was co-chromatographed. Similar results were obtained by 2-dimensional TLC and column chromatography. The solvent front (SF) and origin are indicated.


MARKER
SF
S
ORIGIN
R_f 0.19
MARKER
SF
S
ORIGIN
R_f 0.25
MARKER
SF
S
ORIGIN
R_f 0.62

Florisil and DEAE chromatography.

Mouse spinal cord cultures grown in the presence of 1000 μM Phe and 500 μM PPA were clearly distinguishable from control cultures in being more sparsely myelinated as observed under phase contrast and brightfield microscopy (Figure 10). Cultures grown in the presence of PLA and PA were virtually indistinguishable from control cultures grown under the same conditions. A crude estimation was made of protein synthesis using 1-^{14}C-L-leucine and of incorporation of thymidine [^{3}H-methyl] into DNA in the culture system to compare the control cultures to the PPA-treated cultures. A typical such estimation is shown in Table 3. Any reduction seen in protein synthesis or incorporation of thymidine into DNA was less than 10 percent, and not significantly different from controls.

The Effects of Intracerebral Phe, PPA, PLA, PA, α-KB, α-KIC and α-KIV Injections Upon *In Vivo* Sulfated Galactocerebroside Synthesis in Brain

Phenylalanine, phenylpyruvate, phenyllactate, and phenylacetate injected intracerebrally and bilaterally as described in Methods and Materials produced effects upon sulfated galactocerebroside synthesis not unlike those obtained in the *in vitro* experiments. No significant reduction of incorporation of ^{35}S into sulfated galactocerebrosides occurred when 8 μg (each injection) of either PLA or PA was injected bilaterally and followed by $Na_2{}^{35}SO_4$. The total sulfated

Figure 10. Brightfield photography of mouse CD-1 spinal cord grown in the presence of control (top) phenylpyruvate (center), and phenylalanine (bottom) medium. The [PPA] was 500 µM and [Phe] was 1000 µM. (400X) Cultures were 28 days of *in vitro* age.

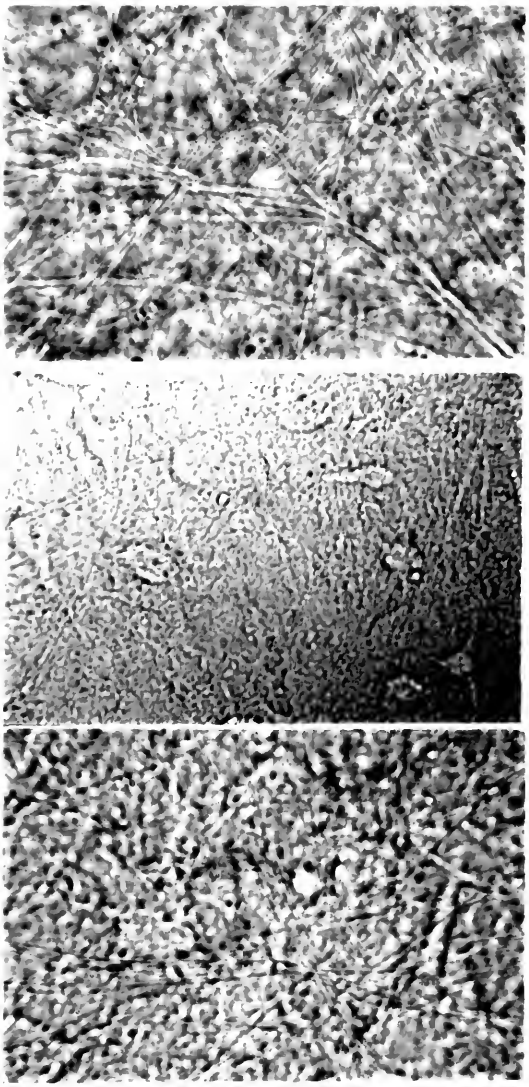


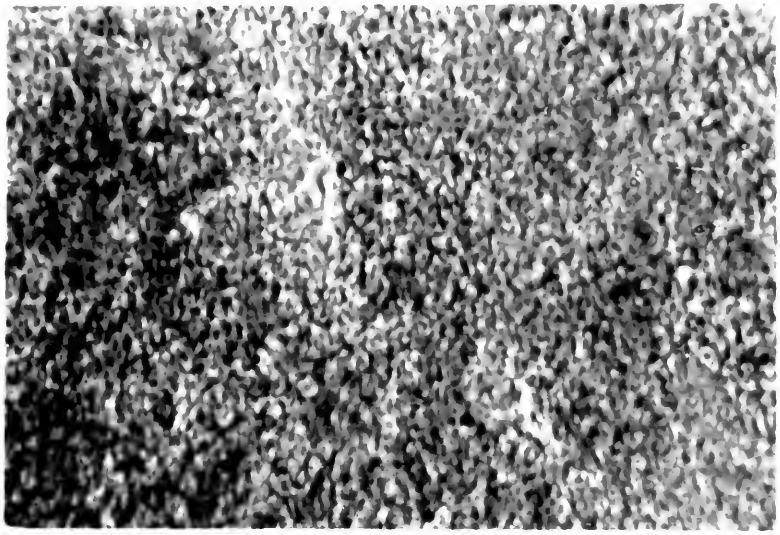
TABLE 3

Crude DNA and Protein Estimation *in vitro* in the Presence and Absence of Phenylpyruvate and Phenylacetate.*

	^{3}H (CPM)	^{14}C (CPM)
Control cord cultures	1225	1791
PPA treated (500uM)	1107	1756
PA treated (250uM)	1297	1815

*EXPERIMENTAL DETAILS ARE DESCRIBED IN MATERIALS AND METHODS.

Figure 11. *In vivo* synthesized myelin in CD-1 mouse spinal cord at 5 days postpartum (top) and 20 days postpartum (bottom).



galactocerebroside content 24 hours after injection has been shown to be for all practical purposes the same for control and treated animals, as shown by sulfate content after hydrolysis by the rhodizonate method described in Methods and Materials. The results of Phe and PPA treatment upon whole brain sulfated galactocerebroside synthesis are shown in Figure 12. Both compounds produced a significant reduction (all $p < .05$) in synthesis in animals 8 to 15 days old. Phe showed less of an effect in adult animals than PPA, and PPA always produced a greater effect in any age mouse used.

Alpha-ketobutyrate and α-ketoisovalerate injection results are shown in Figure 13. In each case, as might be expected, the results were more severe during the period of rapid myelin synthesis. α-KIV injected into adult animals, followed by $Na_2{}^{35}SO_4$ produced control levels of labeled product. The test statistic used was the t-test. The results from all the above experiments are summarized in Table 4.

The Effects of Intracerebral PPA, Phe, α-KB, α-KIC, and α-KIV Injections Upon *In Vivo* Sulfated Galactocerebroside Synthesis in Kidney

Mouse kidney synthesizes sulfated galactocerebrosides *in vivo* [109] as does the rat system discussed by McKhann and Ho [97]. Because of the developmental studies depicting the late appearance of the relevant cerebroside sulfotransferase enzyme in a rodent system described by McKhann and Ho [97], and because of the high activity of the enzyme in kidney, one

Figure 12. *In vivo* whole brain sulfated galactocerebroside synthesis with concomitant bilateral intracerebral injections of saline (control a), phenylalanine (b), and phenylpyruvate (c) as described in the text. An average of 4 animals were used for each data point. Results are expressed as means ± s.e.m.

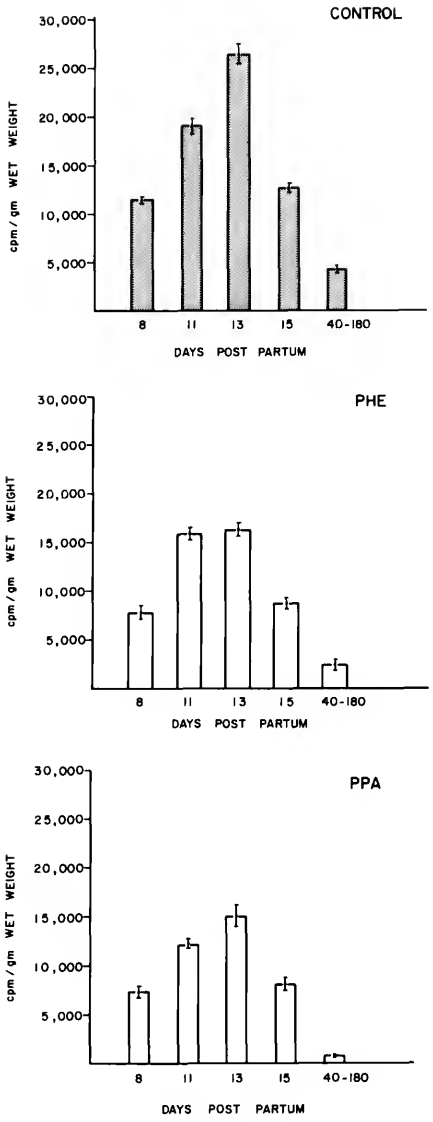
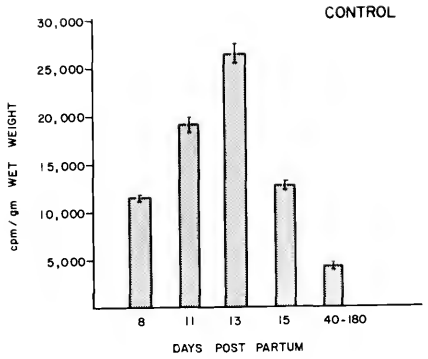

CONTROL
30,000
25,000
20,000
15,000
10,000
5,000
cpm / gm WET WEIGHT
8
11
13
15
40-180
DAYS POST PARTUM
PHE
30,000
25,000
20,000
15,000
10,000
5,000
cpm / gm WET WEIGHT
8
11
13
15
40-180
DAYS POST PARTUM
PPA
30,000
25,000
20,000
15,000
10,000
5,000
cpm / gm WET WEIGHT
8
11
13
15
40-180
DAYS POST PARTUM

Figure 13. *In vivo* whole brain sulfated galactocerebroside synthesis with concomitant bilateral intracerebral injections of saline (control a), α-ketobutyrate (b), and α-ketoisovalerate (c), as described in the text. An average of 4 animals were used for each data point. Results were expressed as means ± s.e.m.


CONTROL
30,000
25,000
20,000
15,000
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5,000
cpm / gm WET WEIGHT
8
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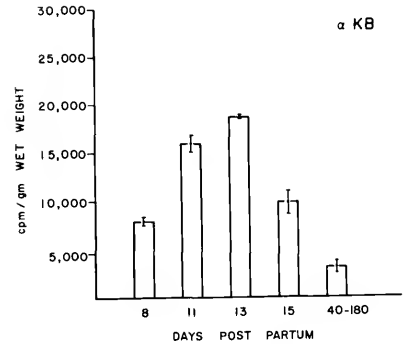
13
15
40-180
DAYS POST PARTUM


α KB
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cpm / gm WET WEIGHT
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DAYS POST PARTUM

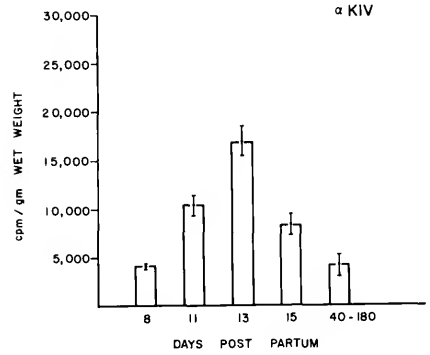

α KIV
30,000
25,000
20,000
15,000
10,000
5,000
cpm / gm WET WEIGHT
8
11
13
15
40-180
DAYS POST PARTUM

TABLE 4

Effect of bilateral intracerebral injections of phenylalanine, phenylpyruvate, phenyllactate, phenylacetate, α-ketobutyrate, α-ketoisocaproate, and α-ketoisovalerate upon brain sulfated galactocerebroside synthesis. Control animals received saline. Average n was 4 animals, except for adults, where 3 mice were used per data point. Details are described in Methods and Materials. Results are reported as mean lipid-soluble ^{35}S counts per gm wet weight whole brain ± s.e.m. N.D., not determined, N.S., not significant at .05 level.

AGE (dpp)	TREATMENT							
	Control	PPA	Phe	PLA	PA	α KB	α K1C	α K1V
8	11,533 ± 372	7,292 ± 579 p < .01	7,949 ± 493 p < .01	N.D.	N.D.	8,943 ± 752 p < .05	7,607 ± 1,012 p < .05	4,199 ± 243 p < .01
11	19,200 ± 956	12,301 ± 833 p < .01	15,970 ± 731 p < .05	19,377 ± 749 N.S.	17,809 ± 570 N.S.	15,949 ± 393 p < .05	N.D.	10,374 ± 1,087 p < .01
13	26,557 ± 1,033	15,155 ± 1,232 p < .001	16,305 ± 656 p < .01	N.D.	N.D.	18,741 ± 747 p < .01	N.D.	17,036 ± 1,571 p < .01
15	12,715 ± 410	8,040 ± 521 p < .001	8,765 ± 545 p < .01	N.D.	N.D.	9,860 ± 827 p < .05	N.D.	8,387 ± 1,115 p < .05
40-180	4,236 ± 376	1,101 ± 321 p < .01	2,462 ± 593 N.S.	N.D.	N.D.	3,063 ± 478 N.S.	3,028 ± 651 N.S.	4,135 ± 909 N.S.

might expect the young adult mouse to be a sensitive system in which to study various chemical effects upon sulfated galactocerebroside synthesis in relation to effects observed in brain.

Adult mice kidneys were removed from animals that had received bilateral intrecerebral injections of PPA, Phe, α-KB, α-KIC, and α-KIV, followed by $Na_2{}^{35}SO_4$ as described previously. After delay period of a few hours, decreased synthesis was found in the kidney. In Figure 14, the significant decrease in incorporation of $Na_2{}^{35}SO_4$ into lipid-soluble sulfated galactocerebrosides in PPA-injected animals is readily apparent. Conversely, α-KIV seemed to have no effect. Phe, α-KB, and α-KIC-treated animals reflected a moderate (nonsignificant at the .05 level) reduction compared to control values. A possible interpretation of these results is presented as they relate to brain synthesis in general discussion.

In Vitro Formation of $^{14}CO_2$ from 1-^{14}C-pyruvate in the Presence of PPA and α-KIC in Mouse Brain, and of PPA and α-KIV in Mouse Kidney

In whole brain homogenates, 1×10^{-3} and 1×10^{-2} M phenylpyruvate produced 28 percent and 47 percent reductions (Figure 15), respectively, in the release of $^{14}CO_2$ from 1-^{14}C pyruvate under the experimental conditions described in Methods and Materials. α-KIC at 1×10^{-3}M reduced by 52 percent the amount of $^{14}CO_2$ released in control homogenates. Such effects

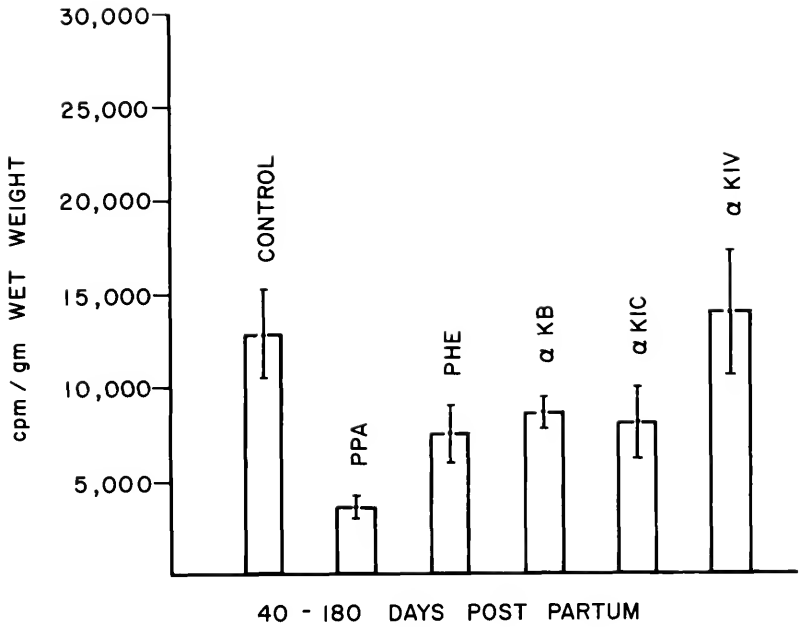


Figure 14. The effect of bilateral intracerebral injections of phenylpyruvate, phenylalanine, α-ketobutyrate, α-ketoisocaproate, and α-ketoisovalerate upon adult mouse kidney sulfated galactocerebroside synthesis. Three adult animals 40 to 180 days of age represent each data point, and the results are expressed as mean cpm ^{35}S as sulfated galactocerebroside per gram wet weight of kidney ± s.e.m. Each animal received 8 µg bilaterally and intracerebrally in a volume of 1 µl of each compound shown, followed by 0.57 µCi $Na_2{}^{35}SO_4$ per gm body weight as described in Methods and Materials. Lipid-soluble sulfated galactocerebroside-specific counts are indicated.

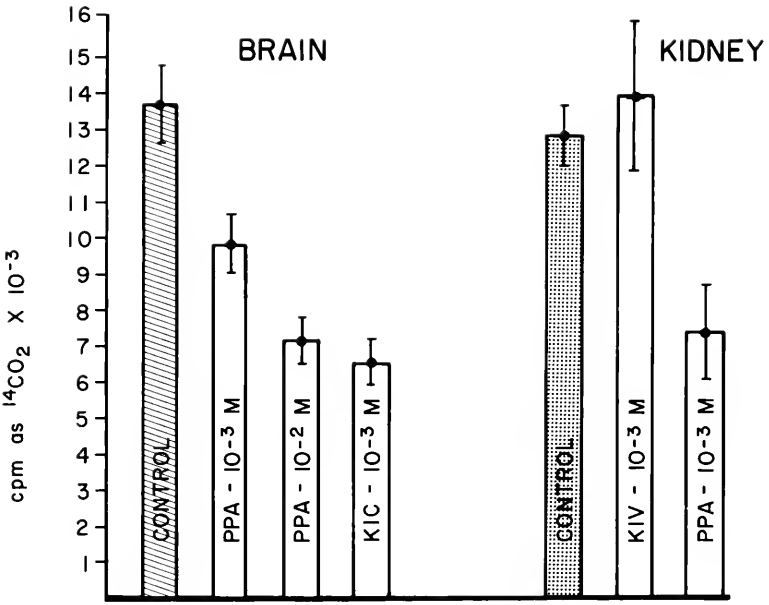


Figure 15. The effects of phenylpyruvate and α-ketoisocaproate upon release of $^{14}CO_2$ from 1-^{14}C labeled pyruvate in CD-1 mouse brain homogenates, and by phenylpyruvate and α-ketoisovalerate in adult mouse kidney homogenates. The homogenates were compared and incubated as described in Methods and Materials. Results are reported as mean cpm as $^{14}CO_2$ ±s.e.m. for each homogenate, with n = 3.

in vivo would be potentially devastating upon energy production and precursor molecule availability for brain lipid biosynthesis. The effects would be compounded first by the heavy demands for ATP and reducing equivalents by the brain during the early spurt of myelin-precursor synthesis, and secondly by the fact that levels of several of the compounds above are unfortunately maintained, and are available to the brain and other organs in several clinical conditions.

In the kidney homogenates, α-ketoisovalerate at 1×10^{-3} M released 108 percent of control levels of $^{14}CO_2$ from 1-^{14}C-pyruvate, but PPA at 1×10^{-3}M produced a 42 percent reduction in $^{14}CO_2$ release as shown in Figure 15.

GENERAL DISCUSSION

Decarboxylation of Pyruvate and Structurally Related Compounds

In the experiments of Burton et al. [109], synthesis of sulfated galactocerebroside and galactocerebroside was paralled by incorporation of labeled galactose, and PAPS synthesis has been observed very early in the newborn rat [110]. It has been suggested as a result of these, and other experiments by one author [97] that the availability of galactocerebroside may be one of the major limiting factors in the synthesis of sulfated galactocerebrosides in the intact animal. The levels of inhibition observed in the decarboxylation of 1-^{14}C pyruvate in this work suggest that the substrate available for sulfation would probably be significantly reduced *in vivo* upon continuous exposure to the compounds demonstrating the inhibition, and secondly that ATP production would be reduced during a critical period of nervous system development. One might expect these effects to bear consequences upon subsequent myelination events as well, such as further cerebroside synthesis and deposition into myelin.

One might predict first, that in the presence of chronically elevated PPA levels in mice for instance, the endogenously bound cerebroside would be reduced, and secondly that such a reduction would be demonstrable in an endogenous

cerebroside sulfotransferase assay as described by Balasubramanian and Bachawat [111]. A second question to be answered is whether or not PPA is actively decarboxylated in the present *in vitro* assay system. Synthesis of 1-^{14}C phenylpyruvate and incubation with the kidney and brain homogenates would provide a clue as to the exact nature of the inhibition observed.

A differential sensitivity of pyruvate dehydrogenase to inhibition by KIC, based upon developmental age, has recently been reported by Bowden et al. [112] in a chick embryo system. The level of inhibition increased sharply at a constant concentration of KIC beginning at day 12. The important point here is that the susceptibility of the pyruvate dehydrogenase system to inhibition by KIC was rapidly rising or high during early myelination. This observation may prove to be more universal in terms of other animal systems as well when investigated more thoroughly. Both KMV and KIC have been shown in rats to be inhibitory upon decarboxylation of pyruvate in brain and liver, and PPA has been recently shown to be inhibitory in rat brain, but not in liver. PLA has no significant effect in either brain or liver [113,114]. Results of this study showed no significant inhibition of decarboxylation in kidney homogenates using adult mice in the presence of 1×10^{-3}M KIV, but found a significant decrease at only 1×10^{-3}M PPA. Both PPA and KIC were inhibitory in CD-1 mouse brain homogenates at 1×10^{-3}M. Of the branched-chain α-keto acids tested in an *in vitro* culture system by Silberberg [115], only KIC inhibited the formation of myelin at

1×10^{-3}M, and it was cytotoxic at 3×10^{-3}M. The present experiment's mortality rates exceeded 50 percent, to as high as 100 percent, when mice 11 to 15 days of age were injected intracerebrally with the same amount of KIC given the mice in the other treatments. (Mortality rates in all other treatment classes were negligible.) The few mice that survived during that period contained less than 1000 dpm as sulfated galactocerebroside per gm wet weight of brain. Such devastating effects in sulfated galactocerebroside net synthesis would also be expected to lead to impaired synthesis and incorporation of cerebroside, basic protein, cholesterol, and other important elements into myelin membrane, and to produce permanent functional deficits.

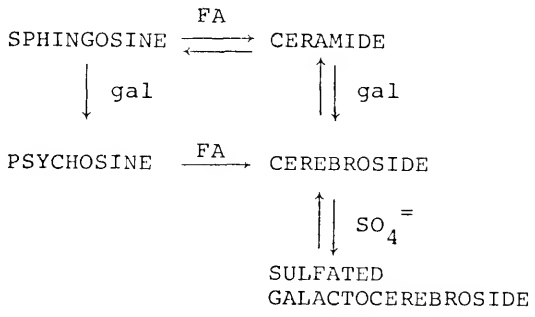
Relationship of Various Sulfated Cerebroside Metabolism Enzymes

A galactocerebroside sulfotransferase has been isolated from rat kidney by McKhann and Ho [97] that has similar properties to the brain enzyme as to pH optimum, specificity, and location within the cell. It is not clear, however, if the two enzymes are identical. Additional links exist between kidney and brain sulfated galactocerebroside synthesis that emphasize the need for additional comparative biochemical studies of the two structures. Sulfated galactocerebrosides present in such large amounts in the myelin of metachromatic leukodystrophy patients also accumulate up to 70 times normal amounts in the kidney [116]. It has been suggested by

Cumar and co-workers [117] and by McKhann and Ho [97] that the kidney sulfatase enzyme for sulfated galactocerebroside and ceramide dihexoside sulfate cleavage is one and the same enzyme. Present evidence to date indicates that arylsulfatase A and cerebroside sulfatase are similar if not identical [118], and histochemical evidence indicates that arylsulfatase A is located very near the myelin sheath in both the CNS and PNS [119]. Further isolation and purification of these enzymes will aid in clarifying their roles in sulfated galactocerebroside metabolism in brain, kidney, and other structures.

Metabolism and Transport of Sulfated Galactocerebroside

Considerable interest has been generated in the assessment of alternative pathways for the synthesis of sulfated galactocerebrosides. A generalized scheme showing both major proposed pathways are shown below:



KEY

FA--fatty acid
gal--galactose

In earlier experiments of Brady [120,121], psychosine was acylated by acyl-CoA to form galactocerebroside. Morell and co-workers [122,123] demonstrated synthesis of galactocerebroside from ceramide and UDP-galactose, and could not acylate psychosine. A recent paper claims enzymatic synthesis of cerebroside, however, from galactosylsphingosine and stearoyl CoA [124] in embryonic chicken brain. Apparently the major pathway in the synthesis of cerebroside is the ceramide pathway. The significance of the psychosine pathway in the synthesis of cerebrosides and sulfated galactocerebrosides is currently under investigation in several laboratories.

A sulfated galactocerebroside-containing lipoprotein fraction was characterized in 1968 by Herschkowitz and coworkers from rat brain [125], and it was suggested that sulfated galactocerebroside is transported from its site of synthesis in the microsomal fraction to the myelin membrane by water-soluble lipoproteins very unlike serum lipoproteins. Little is currently understood about the specific mechanism of transport of myelin precursors from their sites of synthesis to the site of incorporation, and studies of how these processes are controlled are just beginning.

Myelination Sequence

Myelination in the mouse CNS is preceded by a period of rapid proliferation of oligodendroglial elements and cell

membrane at 10 to 11 days of age. Little myelin can be detected at this time [126]. The whole brain DNA content has been shown by Matthieu and co-workers [69] to equal essentially adult values by 12 days postpartum. The large increase in DNA just prior to 12 days reflects mainly oligodendroglial proliferation [127].

Rapid proliferation of glia is proceeded by the formation of a compact myelin membrane. The current experiments have shown peak incorporation of $^{35}SO_4^=$ into sulfated galactocerebroside myelin precursor molecules in whole brain, cerebrum, cerebellum, and spinal cord to occur at this time in CD-1 mice. *In vivo* experiments in this work indicate that mice treated with PPA, Phe, PLA, PA, α-KB, α-KIC, and α-KIV still followed precisely this rigid myelination sequence. It is reasonable to assume that deficient synthesis within this period would compound the error in subsequent formation of compact myelin, and lead to impaired function. Dobbing [128] pointed out the concept of vulnerable periods in developing brain and it seems applicable in the interpretation of the observed inhibition of the incorporation of $^{35}SO_4^=$ into sulfated galactocerebrosides in the present experiments.

Route of Injections in CD-1 Mice

In the *in vivo* experiments of Chase and O'Brien [100] in rats, subcutaneous injections of PPA at 5 gm/kg/24 hours for 18 days followed by i.p. ^{35}S-sulfate resulted in control

values of sulfated galactocerebroside synthesis when brains were extracted 24 hours later. Phenylalanine administered in the same manner and at the same dosage produced approximately a 50 percent decrease in incorporation of ^{35}S sulfate into sulfated galactocerebrosides. In preliminary injections of both i.p. and intracerebral injections of PPA in CD-1 mice in the current experiments, significant reductions in ^{35}S lipid-soluble counts were observed in each case. It is suggested, therefore, that in the rat subcutaneous injections PPA did not reach the blood or that adequate blood levels were not maintained for some reason in the brain. The exact reasons for the difference in results are not clear at this time.

Tissue and Organ Culture of Mouse Brain

Synaptogenesis, differentiation of neurons and glial elements, and myelinogenesis have been recently studied in cerebrum, cerebellum and spinal cord culture [17]. The requirement for basic protein in early myelin synthesis has been demonstrated utilizing antibody to the protein in an *in vitro* culture system [129] by Bornstein and Raine. In the presence of antibody myelination was arrested. After removal of the antibody and rinsing the cultures, myelination continued. This is further evidence to suggest that myelination (at least in some stages) proceeds in an orderly timed sequence, perhaps involving obligate steps for further

synthesis and deposition of myelin into compact mature membrane. Rabbit EAE sera have been shown to contain two dissimilar antibody specificities to cerebroside and encephalitogenic protein. Sera from these rabbits produce demyelination in CNS cultures, given at sufficient levels, and oligodendroglial differentiation and myelin formation are inhibited in mouse spinal cord cultures. Guinea pig serum to basic protein did not inhibit myelin formation in the cultures, but sulfated galactocerebroside synthesis was inhibited using rabbit anti-cerebroside antibody.

Based upon results obtained in the *in vitro* brain culture system, those from the *in vivo* intracerebral injections, and from the experiments on the brain and kidney homogenates, it is consistent with these results to suggest that inhibition of synthesis of sulfated galactocerebroside in these systems was at least related to the degree of inhibition of pyruvate decarboxylation. One would then predict that even in transient conditions in which 2-keto acids inhibitory to pyruvate decarboxylation were elevated in blood that sulfated galactocerebroside synthesis would be reduced, and that should such elevated levels occur during early myelin maturation, irreversible loss of function could result. An important consideration in assessing the reversibility of recovery as observed in sulfated galactocerebroside synthesis in the PPA experiments *in vitro* is discussed by Anderson, Rowe, and Guroff [130] in their paper on behavioral changes in rats with experimental PKU, and that is reversibility or recovery from low levels of synthesis

during a critical period of development does not *a priori* confer the brain with immunity from permanent functional damage, even if the apparent structural recovery seems to be complete upon further development [128].

SUMMARY

Maximum incorporation of radioactive sulfate as $Na_2{}^{35}SO_4$ into sulfated galactocerebrosides in CD-1 mouse cerebrum, cerebellum, and spinal cord occurred at approximately 13 days postpartum *in vivo*, after a rapid onset beginning at day 5 to 8.

In vitro synthesis of sulfated galactocerebroside in mouse spinal cord culture was shown to be inhibited by 1000 µM Phe and 500 µM PPA to the extent of 50 percent and 70 percent, respectively. PLA at 300 µM and PA at 250 µM had no significant effect. The PPA and Phe-treated cultures at high concentrations of 500 µM and 1000 µM, respectively, demonstrated delayed myelination compared to controls of the same age *in vitro*, and such cultures were more sparsely myelinated as seen under phase and brightfield microscopy. The inhibitory effect of PPA at 500 µM upon sulfated galactocerebroside synthesis was demonstrated to be reversible when control medium replaced the PPA-containing medium.

The contribution of methionine sulfur to sulfated galactocerebroside synthesis was investigated, and was found to demonstrate peak incorporation at approximately 14 days postpartum at a level of 2600 dpm per gram wet weight of whole brain when injected i.p., 0.57 µCi at 40 Ci/mM.

Bilateral intracerebral injections of Phe, PPA, α-KB,

α-KIC, and α-KIV at 8 μg each followed by i.p. $Na_2{}^{35}SO_4$, significantly reduced (all $p < .05$) incorporation of $^{35}SO_4{}^{=}$ into sulfated galactocerebroside during the period 8 to 15 days postpartum. PLA and PA injected near the age of maximum incorporation however, resulted in no significant effect upon incorporation of $^{35}SO_4{}^{=}$ into product. In adult mice 40 to 180 days old only PPA had a significant effect of decreased incorporation at the rejection level of .05 ($p < .02$). KIV-treated animals produced control amounts of sulfated galactocerebroside, while Phe, α-KB and α-KIC did show a reduction.

Adult CD-1 mice also reflected a decrease in their kidney sulfated galactocerebroside synthesis with PPA ($p < .02$) at lesser reduction with Phe, α-KB, and α-KIC, and no change with α-KIV.

The effect of PPA as a structural analog of pyruvate was investigated as to its ability to increase or decrease the *in vitro* decarboxylation of pyruvate in kidney and brain homogenates. PPA reduced the amount of $^{14}CO_2$ produced from 1-^{14}C pyruvate by 28 and 47 percent at 1×10^{-3}M and 1×10^{-2}M, respectively. In the kidney homogenates, PPA at 1×10^{-3}M produced a 42 percent reduction in $^{14}CO_2$ released, but α-KIV produced control values under the same conditions.

It is suggested that such severe reductions in CO_2 release from pyruvate in the kidney and brain homogenates in the presence of PPA in the present study, along with the

results of the bilateral intracerebral injections of the other compounds tested *in vivo*, could result in decreased PAPS synthesis (requiring ATP), result in a decrease in endogenously bound galactocerebroside substrate, and result in related oxidative metabolism deficits largely accounting for the observed decreases of incorporation of $^{35}SO_4^{=}$ into sulfated galactocerebroside *in vivo* and *in vitro* in CD-1 mouse kidney and brain.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

O. M. Rennert, Chairman
Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

C. M. Allen, Jr.
Associate Professor of Biochemistry

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J. M. Tsibris
Assistant Professor of Biochemistry

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This dissertation was submitted to the Graduate Faculty of the Department of Biochemistry in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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